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Differential Regulation of the Cellulase Transcription Factors XYR1, ACE2, and ACE1 in *Trichoderma reesei* Strains Producing High and Low Levels of Cellulase▽†

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Due to its capacity to produce large amounts of cellulases, *Trichoderma reesei* is increasingly being investigated for second-generation biofuel production from lignocellulosic biomass. The induction mechanisms of *T. reesei* cellulases have been described recently, but the regulation of the genes involved in their transcription has not been studied thoroughly. Here we report the regulation of expression of the two activator genes *xyr1* and *ace2*, and the corepressor gene *ace1*, during the induction of cellulase biosynthesis by the inducer lactose in *T. reesei* QM 9414, a strain producing low levels of cellulase (low producer). We show that all three genes are induced by lactose. *xyr1* was also induced by D-galactose, but this induction was independent of D-galactose metabolism. Moreover, *ace1* was carbon catabolite repressed, whereas full induction of *xyr1* and *ace2* in fact required CRE1. Significant differences in these regulatory patterns were observed in the high-producer strain RUT C30 and the hyperproducer strain *T. reesei* CL847. These observations suggest that a strongly elevated basal transcription level of *xyr1* and reduced upregulation of *ace1* by lactose may have been important for generating the hyperproducer strain and that thus, these genes are major control elements of cellulase production.

Recent demands for the production of biofuels from renewable carbon sources have led to increased interest in the hydrolysis of cellulose, a β-(1,4)-linked glucose polymer which is the product of the utilization of solar energy and carbon dioxide by plants and exhibits an annual production of about 7.2×10^{10} tons. Its degradation is a key transformation step in the biological carbon cycle in nature and also in the production of second-generation biofuels (8). *Trichoderma reesei*, the anamorph of the tropical ascomycete *Hypocrea jecorina*, is one of many saprobic fungi that are capable of efficiently degrading plant cell wall polysaccharides, such as cellulose or hemicelluloses. Possibly due to an intriguing clustering of cellulase genes in its genome (21), *T. reesei* is superior in its ability to form and secrete cellulases and is the major fungus for industrial cellulase production (2, 17, 25).

The *T. reesei* genome encodes 10 cellulases and 16 hemicellulases (21). From a biological perspective, the expression of these genes must be tightly regulated, because the synthesis of the proteins encoded and their secretion requires a great deal of energy. They are therefore synthesized only in the presence of an inducer, which can be cellulose itself or disaccharides generated by its degradation (such as sophorose) (4, 16). Also, the galactosyl-β-1,4-glucoside lactose is a strong inducer and virtually the only soluble inducing carbon source for commercial cellulase production (16).

Most of the cellulase genes are regulated in a consistent manner (11), suggesting a fine-tuned cooperation of the respective transcription factors (TFs). At present, five such TFs have been identified: the positive regulators XYR1, ACE2, and the HAP2/3/5 complex, as well as the repressor ACE1 and the carbon catabolite repressor CRE1 (16). XYR1 (xylanase regulator 1) is considered the main activator of cellulase and hemicellulase gene expression (35, 36), because its deletion eliminates cellulase induction by all inducers and also impairs the induction of different hemicellulase genes involved in xylan and arabinan degradation (1, 35). It is an orthologue of the *xlnR* gene of *Aspergillus niger*, which controls the transcription of more than 20 genes encoding hemicellulases and cellulases (36). Deletion of the second cellulase activator-encoding gene characterized, *ace2*, lowered the transcript levels of the major cellulases and reduced cellulase activity to 30 to 70% when the fungus was grown on cellulose but had no effect on cellulase induction by sophorose (5). Interestingly, XYR1 and ACE2 are able to bind to the same promoter motif [GGC(T/A)$_4$] (12). Stricker et al. (38) suggested that phosphorylation and dimerization are prerequisites for the binding of ACE2 to its

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† Supplemental material for this article may be found at http://ec.asm.org/.

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TABLE 1. *Trichoderma reesei* strains

Name or genotype	Reference
QM 9414	Mandels and Andreotti, 1978 (20)
Δ*cre1*	This study
Δ*xyr1*	Stricker et al., 2006 (35)
Δ*gal1*	Seiboth et al., 2004 (30)
Δ*xyl1*	Seiboth et al., 2007 (29)
Δ*gal1* Δ*xyl1*	Seiboth et al., 2007 (29)
RUT C30	Eveleigh and Montenecourt, 1979 (10)
CL847	Durand et al., 1988 (9)

target promoter. The HAP2/3/5 complex is believed to be necessary for the generation of an open chromatin structure, necessary for full transcriptional activation (41). The repressor role of ACE1 has been suggested by studies demonstrating that deletion of *ace1* results in an increase in the expression of all the main cellulase and hemicellulase genes in sophorose- and cellulose-induced cultures (3). ACE1 also represses *xyr1* expression during growth on D-xylose (19). CRE1 is the main transcription factor mediating carbon catabolite repression (CCR) (14, 34), a mechanism that favors the assimilation of high-energy-yielding carbon sources over that of sources yielding less energy. CRE1 is impaired in strain RUT C30 (14), which produces very high levels of cellulase (hyperproducer), and the importance of this gene for the improvement of enzyme production in *T. reesei* has been emphasized recently (24).

TFs are often present in cells only in small amounts, and they function as pacemakers for the processes in which they are involved (27). Many of them, therefore, are further induced by the conditions for which they are needed and are degraded once they are no longer needed (40). In contrast, *xyr1* expression has been reported to be regulated solely by CRE1-dependent CCR and by repression by the specific transcription factor ACE1, not by induction (19, 37). Such a mechanism, if correct, would tightly link *xyr1* expression to the flux through carbohydrate catabolism but would preclude the possibility of responding to the presence of a potential inducer.

The objective of this paper was therefore to reassess how *xyr1* and two other putatively specific cellulase-related TFs (i.e., ACE1 and ACE2) are regulated under conditions of cellulase induction and how this regulation relates to carbon catabolite repression. Lactose was chosen as the cellulase-inducing carbon source, because growth on lactose is independent of cellulase activity. In addition, we compared the expression patterns of these TFs with those observed in a high-producer strain in the public domain (RUT C30) and an industrial hyperproducer strain (CL847). Our findings offer new insights into the mechanism of cellulase induction, as well as targets for the improvement of *T. reesei* strains.

MATERIALS AND METHODS

Fungal strains. The *T. reesei* strains used in this study and their references are given in Table 1. All strains were maintained on malt extract agar. Spores were resuspended in a sterile NaCl (9 g · liter^{-1})–20% (wt/vol) glycerol solution and were stored at −80°C. A *cre1* deletion vector was constructed by using the double joint PCR technique (39). The oligonucleotides used for the construction of the deletion fragment and the Δ*cre1* strain are described in the supplemental material.

Cultivation conditions. Frozen spores were used to inoculate a Fernbach flask containing 250 ml of the culture medium described previously (13). Cultivation was carried out at 30°C with stirring at 150 rpm. After 72 h, the medium containing mycelia was used as an inoculum for bioreactor culture.

Cellulase was produced in a 4-liter bioreactor with a two-step cultivation procedure. Strains were first grown at 28°C in 2 liters of a medium containing 30 g · liter^{-1} of glucose as a carbon source and pH regulated at 4.8 with 5.5 M NH_3. The airflow was adjusted at 0.5 volume per volume per min (VVM), and initial stirring was set at 400 rpm. This parameter was gradually increased to maintain partial O_2 pressure (pO_2) above 40% oxygen saturation. When the initial glucose was close to depletion (<20% of initial glucose content), the fed-batch phase was initiated. During this second step, a 250-g · liter^{-1}carbon source solution (either glucose or lactose) was injected at a rate of 0.98 g · h^{-1}. Samples were collected periodically to determine the biomass, carbon, and protein concentrations.

D-Galactose pathway deletion strains were cultivated in shake flasks as described previously (29). For transfer cultures, strains were pregrown for 24 h in 1-liter flasks on a rotary shaker (250 rpm) at 30°C in 250 ml of the medium described by Mandels and Andreotti (20) with glycerol (1% [wt/vol]) as a carbon source. They were then harvested, washed with a medium without a carbon source, and transferred to a fresh medium containing D-galactose or D-glucose as a carbon source (1% [wt/vol]), as specified in Results. Samples for mRNA extraction were then harvested after 2, 4, and 6 h.

Analytical methods. Biomass concentrations were determined by gravimetric analysis. Ten milliliters of the collected culture medium containing mycelia was filtrated on 1.2-μm-pore-size GF/C glass microfiber membranes. Dry biomass was measured 24 h after incubation of the membrane at 105°C.

The glucose concentration during the batch phase was assessed by enzymatic reaction using an Analox GM10 glucose analyzer (Imlab) to predict the convenient time to start the fed-batch phase.

The concentrations of all carbon sources were quantified *a posteriori* by high-performance liquid chromatography (HPLC) using a HPX-87P column (Bio-Rad) maintained at 85°C. He-degassed distilled water was used as an eluant at a flow rate of 0.4 ml · liter^{-1}.

Concentrations of extracellular protein were quantified by the Quick Start Bradford protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard.

RNA sample preparation. RNA samples for analyses of gene expression were prepared from mycelium powder obtained by grinding the filtrated biomass from bioreactor cultivations—or from shake flasks for D-galactose pathway deletion strains and transfer cultures—in liquid nitrogen. The powder was subjected to a phenol treatment using TRI reagent solution (Applied Biosystems). The extracted total RNA was then isolated with bromochloropropane, precipitated with isopropanol, washed with ethanol, and solubilized in nuclease-free water according to the manufacturer's instructions. Samples were cleaned up by following the Qiagen RNeasy procedure and were subjected to on-column DNase digestion with the RNase-Free DNase set (Qiagen).

Transcript analysis by quantitative PCR (qPCR) analysis. Five hundred nanograms of RNA was reverse transcribed (RT) using the iScript cDNA synthesis kit (Bio-Rad) containing a blend of oligo(dT) and random hexamer primers.

qPCRs were performed in an iCycler iQ real-time detection system (Bio-Rad). Each reaction mixture contained 1 μl of the template (1/10 of the previous RT reaction product), 12.5 μl of iQ SYBR green Supermix 2× (Bio-Rad), 0.8 pmol · μl^{-1} forward and reverse primers, and nuclease-free water to a final volume of 25 μl. The PCR protocol consisted of 3 min of initial denaturation at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. A melting curve was performed after each run to check PCR product specificity.

The oligonucleotide primers used are given in Table 2. To ensure the absence of genomic DNA, control samples were subjected to the procedure described above, but no reverse transcriptase was added, and PCRs without a template were set up to rule out contamination of other PCR components. All PCRs were carried out in triplicate within a plate, and every condition was tested in at least two different plates. Identical samples were present on every plate; this calibrator was used to counteract the effects of potential interrun variations for all other samples.

Data were analyzed with iQ 5 Optical System software (Bio-Rad). Using the PCR baseline-subtracted mode, the detection threshold level was set automatically by the software, and the amplification efficiency for each gene was determined. All threshold cycle (C_T) values of a plate were adjusted so that C_T value differences between interrun calibrators were eliminated. In order to compare different samples, the threshold cycles for all genes investigated were corrected with a factor for *gpd1* amplification, as described previously (33).

Expression ratios were analyzed on samples collected just before the beginning of the fed-batch phase (BFB) or 1 h, 6 h, and 24 h after the beginning of extracellular protein accumulation during the fed-batch phase. Since strain QM 9414 started its protein production 18 h after the beginning of the fed-batch

TABLE 2. qPCR oligonucleotides used in this study

Transcript studied	Probe type	Sequence
gpd1	Forward	GGAGCTCTTTGAAGAGGA
gpd1	Reverse	GGCAGGTACTTGACGTTTTC
xyr1	Forward	CCATCAACCTTCTAGACGAC
xyr1	Reverse	AACCCTGCAGGAGATAGAC
cbh1	Forward	CCGAGCTTGGTAGTTACTCTG
cbh1	Reverse	GGTAGCCTTCTTGAACTGAGT
bga1	Forward	GTACAAGGTTGACTCCCTTC
bga1	Reverse	CCATAGTCAGAGCCGTAGAG
ace1	Forward	GGACGAGGAGGAGATTATG
ace1	Reverse	GTGAGTCTTCTCGTGCTT
ace2	Forward	GACAAGAAGCTCAGGTGTC
ace2	Reverse	ACTGTGTTCATGGCTGTG

phase (as can be seen in Fig.1Ai), which corresponds to the time needed to release CCR, transcript expression levels during the fed-batch phase were assessed 1 h, 6 h, and 24 h after the beginning of the fed-batch phase for the Δ*cre1*, CL847, and RUT C30 strains and 19 h, 24 h, and 42 h after the beginning of the fed-batch phase for the parental strain QM 9414. The means ± standard deviations (SD) of replicates are shown in the figures. For each transcript, the lowest ratio measured among strains and conditions was set to 1, and all other ratios were related to it, in order to allow better comparisons.

Statistical analyses. Student *t* tests were performed on all sets of data. All the differences mentioned fulfill the criterion of a *P* value of 0.05.

RESULTS

Accumulation of *cbh1* and *bga1* transcripts during the growth of *T. reesei* QM 9414 on lactose and D-glucose. In order to investigate the transcriptional regulation of the cellulase regulatory genes by lactose, we set up a fed-batch system in which mycelia were first cultivated on D-glucose (batch phase) and then—when the concentration of D-glucose had decreased below 20% of the initial content—were fed with lactose at a rate of 0.98 $g \cdot h^{-1}$ (fed-batch phase). The suitability of this system for the investigation of the secretome in *T. reesei* has been shown recently (13). A D-glucose feed at the same rate was used as a control.

Figure 1Ai shows the pattern of growth, the carbohydrate consumption, and the specific protein production rate in the low cellulase producer *T. reesei* QM 9414. D-Glucose was consumed within 30 h of cultivation, and secretion of cellulases and hemicellulases started around 18 h after the beginning of the fed-batch phase. With this protocol, more than 80% of the extracellular protein comprised cellulase and hemicellulase proteins (13). The concentrations of dry biomass reached 8 to 12 $g \cdot liter^{-1}$ during the batch phase and stabilized after the beginning of protein production.

In contrast, almost no protein was secreted with a D-glucose feed (Fig.1Bi), although dry mass concentrations and D-glucose consumption were similar during the batch phase.

The accumulation of the *cbh1* and *bga1* transcripts, encoding the cellobiohydrolase 1 CEL7A and the extracellular β-galactosidase BGA1, respectively, correlated with the utilization of the fed lactose (Fig. 1Aii and iii). The expression of *cbh1* and *bga1* at the onset of lactose feeding was very low (before the beginning of the fed-batch phase [BFB]) but increased considerably during the fed-batch phase (1 h, 6 h, and 24 h). In contrast, *cbh1* and *bga1* expression was much weaker with D-glucose feeding (Fig. 1Bii and iii). These data demonstrate that the experimental system chosen is well suited to document the induction of *cbh1* and *bga1* by lactose and thus is appropriate for the study of the transcriptional regulation of the cellulase regulatory proteins by lactose.

***xyr1* expression is induced by lactose in *T. reesei* QM 9414.** Having set up an appropriate cultivation system for cellulase induction, we tested the expression of *xyr1*, encoding the major transcriptional regulator of cellulase and hemicellulase formation. As can be seen in Fig. 2, the abundance of the *xyr1* transcript in strain QM 9414 was low at the end of the batch phase but increased about 250-fold during lactose feeding. During feeding with D-glucose, however, only a small increase in *xyr1* expression was observed and its final level was only about 5% of that observed on lactose. These data are in accordance with the assumption of a specific role for lactose in *xyr1* induction.

***xyr1* is positively regulated by induction and by the carbon catabolite repressor CRE1.** Stricker et al. have recently postulated that *xyr1* is not regulated by induction but solely by carbon catabolite (de)repression (37). In order to rule out the possibility that the enhanced expression of *xyr1* on lactose is due to relief of carbon catabolite repression on this carbon source, we used a Δ*cre1* strain of *T. reesei* QM 9414, cultivating it under the same conditions as those described above for QM 9414. The carbon catabolite-derepressed strain indeed accumulated higher *cbh1* mRNA levels at the onset of lactose feeding (see Table S2 in the supplemental material), a finding consistent with previous data showing stimulation of *cbh1* gene expression in a carbon catabolite-derepressed background.

xyr1 transcript accumulation, however, was lower in the Δ*cre1* strain: only at the end of the batch phase was the abundance higher (about 10-fold) than that in QM 9414 (Fig. 2), challenging the repressing role of CRE1/CCR on *xyr1* (37). Feeding of D-glucose to the Δ*cre1* strain increased the relative *xyr1* transcript abundance to the level seen in the parent strain QM 9414, whereas feeding of lactose gradually increased *xyr1* transcript abundance to a final level of 6-fold relative to the BFB level. However, this final transcript abundance was only one-third of that in the parent strain. We therefore conclude that *xyr1* is induced by lactose and that full induction requires the positive action of CRE1.

Lactose induction of *xyr1* is independent of D-galactose metabolism. The induction of cellulases by lactose has previously been shown to require metabolism of lactose through the Leloir pathway and through the alternative reducing pathway of D-galactose catabolism (29, 32). We therefore tested whether *xyr1* induction by lactose in strain QM 9414 would also require this metabolism. To this end, we used isogenic QM 9414 strains bearing knockouts in *gal1* (galactokinase; blocking the Leloir pathway [30]), *xyl1* (aldose reductase; blocking the alternative pathway [29]), and both genes (*gal1 xyl1* [29]). The results (Fig. 3A) show that none of these pathway mutations impaired *xyr1* gene expression on lactose, which apparently occurs even in the absence of D-galactose metabolism.

Since lactose metabolism in *T. reesei* occurs by extracellular hydrolysis of lactose to D-glucose and D-galactose (31, 32), the findings described above raise the question of whether the hydrolysis product D-galactose would also act as an inducer of *xyr1*. Since D-galactose is a repressing carbon source and is able to induce cellulases only at low growth rates (15), we compared

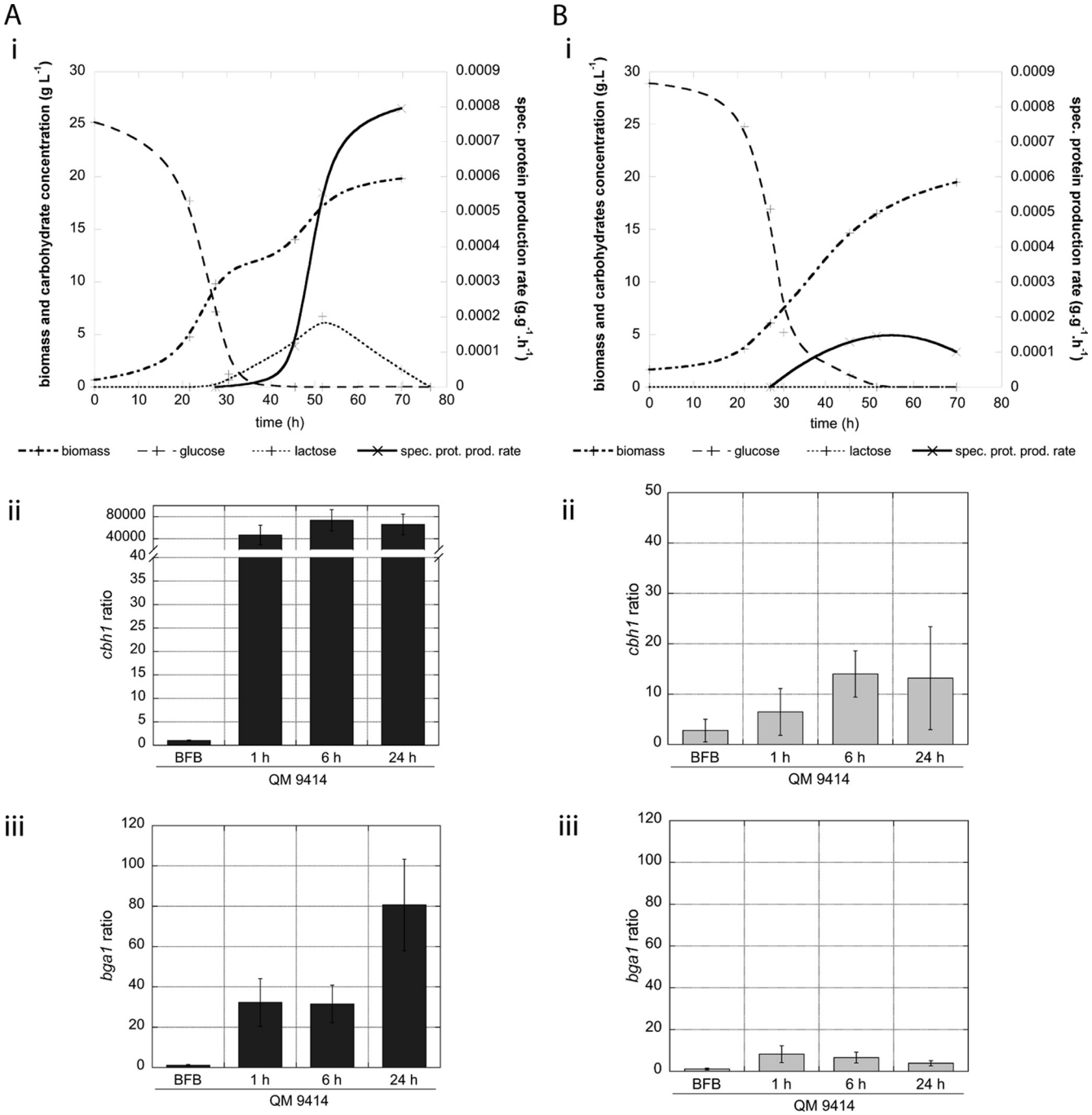

FIG. 1. *T. reesei* QM 9414 accumulates CEL7A and BGA1 when cultivated under a lactose-feeding regimen. After a phase of growth on glucose, *T. reesei* QM 9414 was subjected to a lactose (A) or a glucose (B) feed. (i) The dry biomass concentration, carbohydrate consumption, and specific (spec.) protein production rate were assessed throughout the cultivations. (ii and iii) The ratios of the expression of the *cbh1* (ii) and *bga1* (iii) transcripts to that of the steady-state *gpd1* reference gene were calculated just before (BFB) and 1 h, 6 h, and 24 h after the beginning of the fed-batch phase.

xyr1 expression in shake flasks on D-glucose and D-galactose as carbon sources. Indeed, we found 5- to 10-fold-higher expression of *xyr1* with D-galactose than with D-glucose and glycerol cultures (Fig. 3B). The degree of induction by D-galactose was actually lower than that by lactose, but this is consistent with its action as a carbon catabolite-repressing carbon source, although some of the quantitative differences in transcript abundance may also be due to the different culture conditions used.

***ace2* and *ace1* are also induced by lactose.** Having established that *xyr1* is regulated by lactose, we investigated whether the coactivator gene *ace2* and the repressor gene *ace1* would also be induced by lactose. As shown in Fig. 4A, the *ace2*

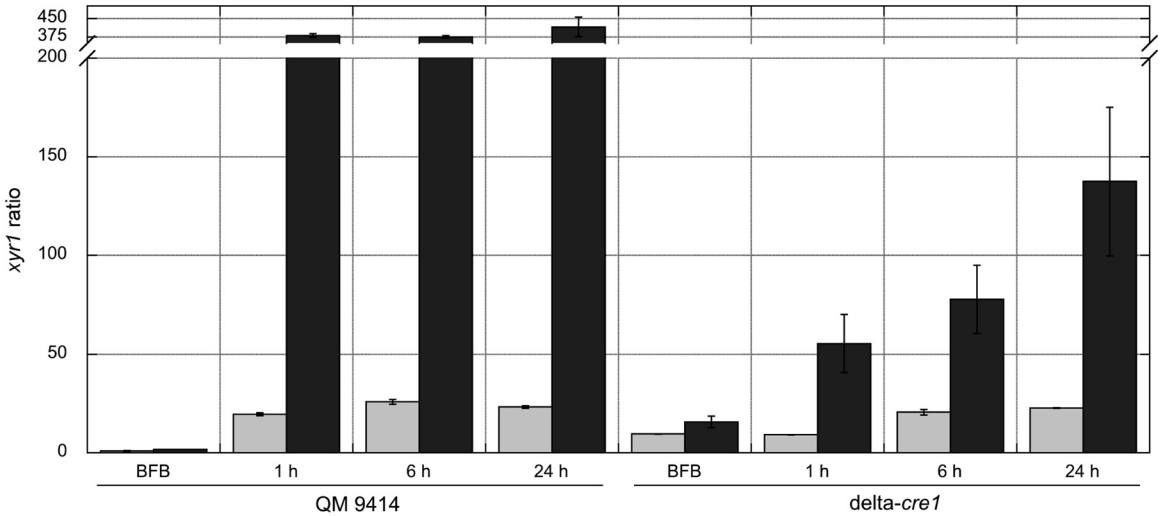

FIG. 2. *xyr1* expression is induced by lactose independently of the release of carbon catabolite repression. The ratio of the expression of the *xyr1* transcript to that of the steady-state *gpd1* reference gene was calculated in *T. reesei* QM 9414 or in a Δ*cre1* strain of QM 9414 fed with glucose (shaded bars) or with lactose (filled bars) after a 25-h growth phase on glucose. Ratios before (BFB) and at different time points during the fed-batch phase are shown.

transcript was present during growth on D-glucose, suggesting partial constitutive expression. Lactose feeding led to a further increase (about 3-fold) in *ace2* transcript abundance. While this is much lower than lactose induction of *xyr1*, it nevertheless indicates lactose induction of *ace2*. Expression of *ace2* was not subject to carbon catabolite repression, as evidenced by the fact that no increased expression levels were observed in the Δ*cre1* strain. In contrast, both the transcript abundance on D-glucose and the transcript abundance upon induction by lactose were in fact lower in the Δ*cre1* strain, suggesting that CRE1 is also required—either directly or indirectly—for full *ace2* expression.

The *ace1* gene, encoding a corepressor of cellulase gene transcription, was also expressed during batch growth on glucose. Its expression remained at the same level during glucose feeding. However, expression also increased moderately upon the shift to lactose feeding (Fig. 4B). Both the basal and the inducible expression levels of *ace1* were about doubled in the Δ*cre1* strain, proving that they are subject to CCR.

Expression of *xyr1* and *ace1* is altered in the *T. reesei* hyperproducer strain CL847. Having assessed the regulatory patterns of the three specific transcriptional regulators, XYR1, ACE2, and ACE1, in the low cellulase producer mutant QM 9414, we wondered whether their expression and/or regulation would have become altered in the course of mutagenesis for high cellulase production. To explore this question, we chose

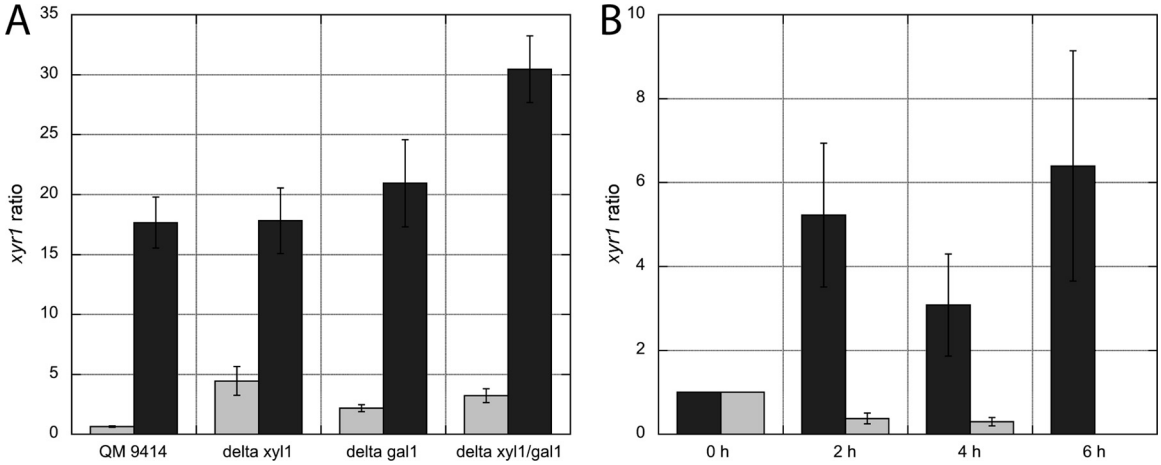

FIG. 3. Induction of *xyr1* by lactose is not dependent on D-galactose metabolism. The ratio of the expression of the *xyr1* transcript to that of the steady-state *gpd1* reference gene was calculated. (A) *xyr1* transcript expression ratios in QM 9414 and galactose pathway mutant strains upon incubation for 20 h (shaded bars) or 30 h (filled bars) on lactose. (B) *xyr1* transcript expression ratios in strain QM 9414, pregrown in shake flasks with glycerol as a carbon source and then transferred to D-galactose (filled bars) or D-glucose (shaded bars) for 2, 4, or 6 h as indicated (only the ratio for galactose was measured at 6 h). The ratio obtained on glycerol (24 h of precultivation) was set to 1.0, and all other ratios are given relative to this value (*n*-fold).

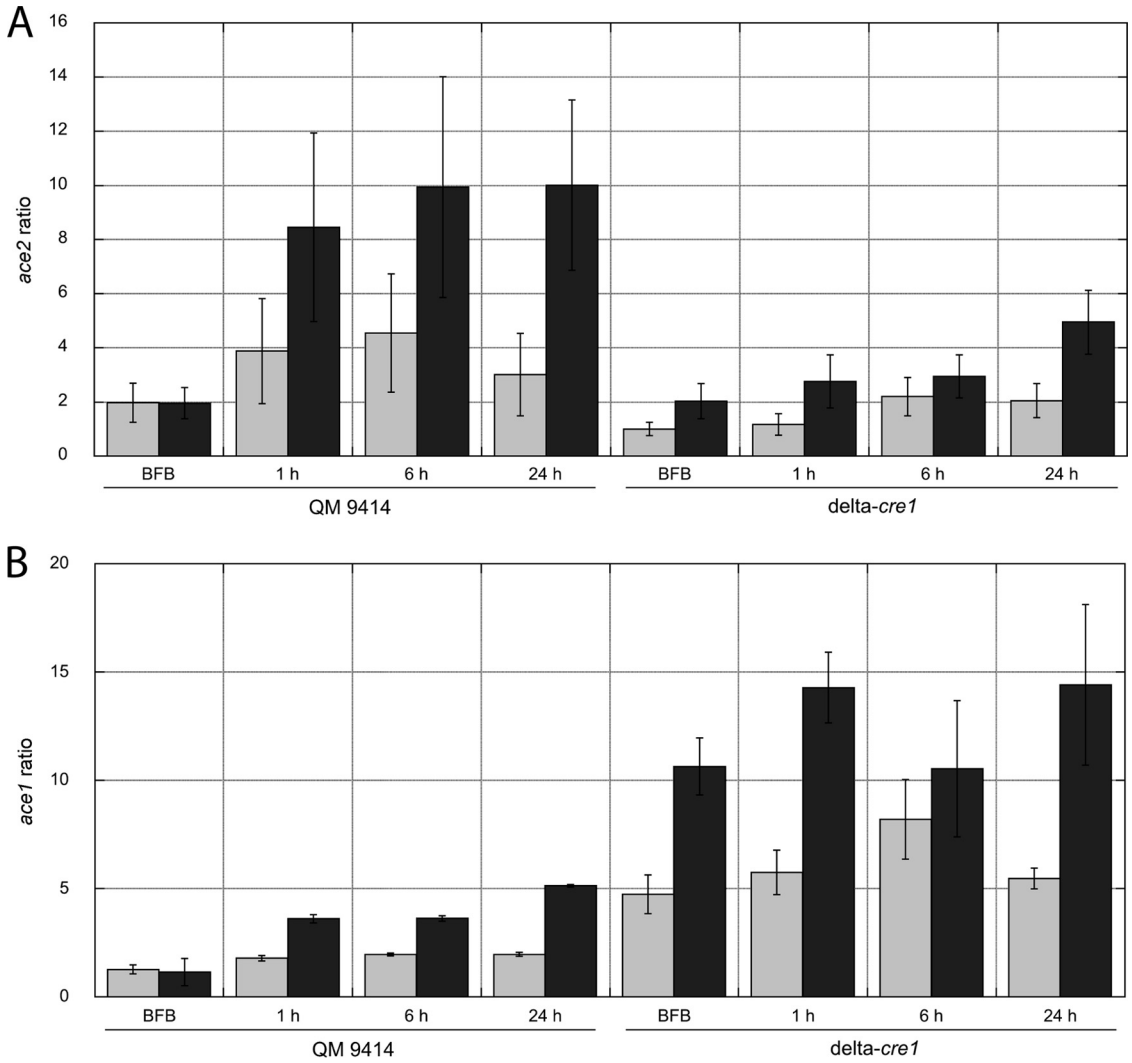

FIG. 4. *ace2* and *ace1* are both regulated by lactose, but only *ace1* is subject to CCR. The ratios of the expression of the *ace2* (A) and *ace1* (B) transcripts to that of the steady-state *gpd1* reference gene were calculated in *T. reesei* QM 9414 and in a Δ*cre1* strain of QM 9414 fed with glucose (shaded bars) or lactose (filled bars) after a 25-h growth phase on glucose. Ratios before (BFB) and at different time points during the fed-batch phase are shown.

the mutant strain CL847 (9), which is the highest cellulase producer strain published so far, and—for analysis of *xyr1*—also the high-producer strain RUT C30 (10, 23). Strain RUT C30 is known to bear a truncated version of the *cre1* gene and is thus carbon catabolite derepressed. Since the mutagenesis scheme published by Durand et al. (9) also includes a screen for 2-deoxyglucose resistance to eliminate CCR, we tested whether CL847 still contains an intact *cre1* locus. To our surprise, it contains the same truncated *cre1* locus as strain RUT C30 (see Fig. S1 in the supplemental material), suggesting that it is a mutant derived from RUT C30. This assumption is further supported by genome sequencing of CL847 (A. Margeot and S. Le Crom, unpublished data). We also sequenced the loci of *xyr1*, *ace1*, and *ace2* in CL847 and found that neither their promoter regions nor their open reading frames (ORF) bear any mutations (see Fig. S2 to S4 in the supplemental material). We also have evidence that all three genes occur as single copies in the CL847 genome (data not shown).

We cultivated CL847 and RUT C30 under the same conditions as those used for strain QM 9414. As shown in Fig. 5, D-glucose consumption and biomass accumulation by CL847 during the batch phase were similar to those for QM 9414. The concentrations of protein excreted into the culture medium were 5- to 15-fold higher than those for QM 9414 at comparable time points (e.g., 0.08 g · liter^{-1} in QM 9414 versus 1.33 g · liter^{-1} in CL847 after 24 h of induction; 0.58 g · liter^{-1} in QM 9414 versus 4.23 g · liter^{-1} in CL847 after 50 h of induction).

The relative transcript abundance of *cbh1* in strain CL847 was already very high before the beginning of lactose feeding. After 24 h, the relative transcript abundance of *cbh1* in CL847 was 10-fold higher than that in QM 9414 (Fig. 6A). The expression of *xyr1* was also already significantly higher at the onset of the feed, and fed-batch levels were 6- to 7-fold higher than those in QM 9414 after 24 h of lactose feeding. During cultivation on D-glucose, the abundance of *xyr1* mRNA in

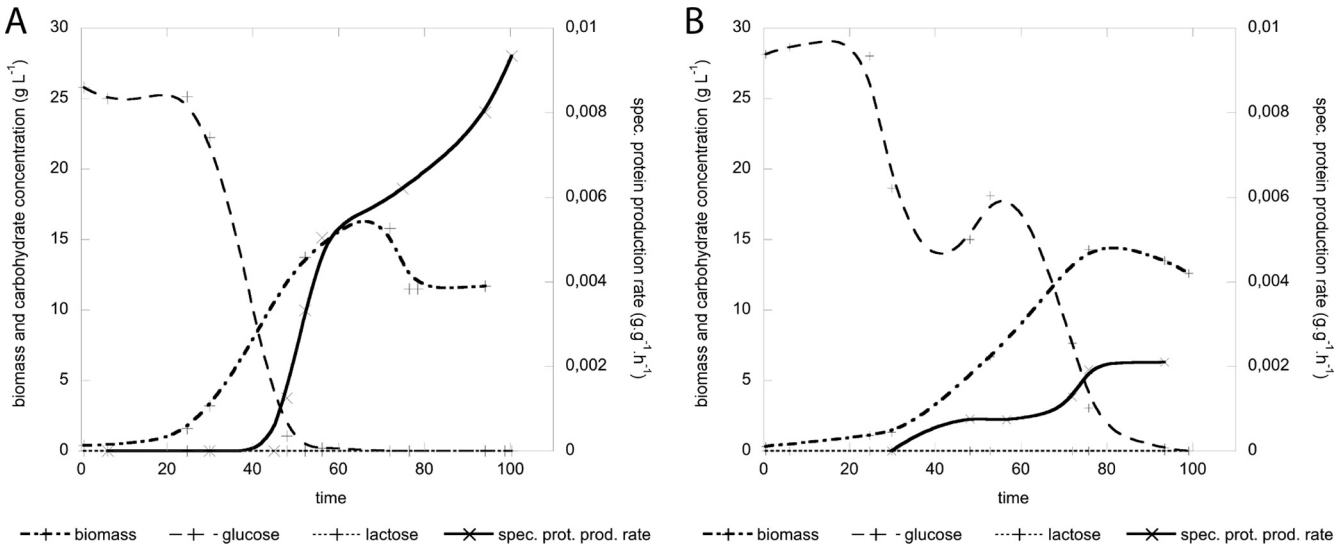

FIG. 5. The *T. reesei* hyperproducer strain CL847 produces cellulases when cultivated under a lactose-feeding regimen. After a phase of growth on glucose, *T. reesei* CL847 was subjected to a lactose (A) or a glucose (B) feed. The dry biomass concentration, carbohydrate consumption, and specific protein production rate were assessed throughout the cultivations.

CL847 was 8-fold higher than that in the Δ*cre1* strain at the beginning, and the *xyr1* mRNA in the CL847 strain remained at about this level during the D-glucose fed-batch process (Fig. 6B). In contrast, *xyr1* transcript abundance in strain CL847 after 24 h was almost 15-fold higher than that at the onset of the lactose feed.

Having identified the *T. reesei* high-producer mutant RUT C30 as an ancestor of CL847 (see above), we were interested in whether *xyr1* transcription had already become elevated in this strain, too. As shown in Fig. 6B, the transcript abundance of *xyr1* in RUT C30 was at about the same level as that in QM 9414, both for basal and for lactose-induced gene expression. The high upregulation of *xyr1* is thus specific for strain CL847.

The expression of *ace2* was less affected in CL847: the basal expression level resembled that observed in the Δ*cre1* strain and thus probably is due to the nonfunctional CRE1 protein in this mutant. Induction by lactose, however, led to about the same expression level as that in strain QM 9414, indicating that the loss of CRE1 does not affect inducible transcription of *xyr1* (Fig. 6C). The basal expression of *ace1* in strain CL847 also resembled that in the Δ*cre1* strain and can be explained by the relief from CCR. Interestingly, *ace1* expression decreased during glucose feeding. Expression on lactose was also higher than that for QM 9414, but well below the levels seen in the Δ*cre1* strain and—notably–was not further induced by lactose during feeding (Fig. 6D).

DISCUSSION

XYR1 is the only transcription factor identified so far in *T. reesei* whose knockout results in the loss of the formation of all cellulases, xylanases, and two arabinofuranosidases (1), as well as in that of some of the enzymes required for the metabolism of the hemicellulose monomers D-xylose, L-arabinose, and D-galactose (29, 35). XYR1 is therefore currently considered to be the main regulator of the cellulase/hemicellulase system of *T. reesei*. Consequently, regulation of *xyr1* expression has a significant impact on the ability of *T. reesei* to produce the various carbohydrate depolymerases and to grow on plant cell wall polysaccharides. In agreement with previous data (19), we found that only a very low level of *xyr1* transcript was formed during growth on D-glucose. However, in contrast with the claim that *xyr1* is not regulated by induction, we found strong induction of *xyr1* by lactose and its hydrolysis product D-galactose. Interestingly, Mach-Aigner et al. also reported an increase in the abundance of the *xyr1* transcript during incubation with another hemicellulose monomer, D-xylose, but interpreted this increase as due to carbon catabolite derepression (19). We consider this interpretation flawed, because D-xylose has been shown to be a carbon catabolite-repressing carbon source in *Saccharomyces cerevisiae* (6) and *Aspergillus nidulans* (26). We admit that proof for D-xylose as a carbon catabolite-repressing carbon source in *T. reesei* has not yet been published, but its operation can be deduced from the fact that cultivation of *T. reesei* on D-xylose does not even lead to the low cellulase levels that are formed during carbon catabolite derepression (28). We therefore speculate that the data of Mach-Aigner et al. are in fact consistent with our conclusion that *xyr1* is regulated by a feed-forward activating circuit similar to that seen with *bga1* and *xyl1* (29, 31). In summary, our results indicate that *xyr1* expression is increased by lactose induction. In addition, our data show that the carbon catabolite repressor CRE1 is—in contrast—required for full induction by lactose, rather than repressing its expression. A positive effect of *A. nidulans* CreA on gene expression has been reported previously (22) and is also observed for several other *T. reesei* genes (T. Portnoy et al., submitted for publication).

The transcriptional regulation of the other two putative specific regulators also revealed induction by lactose, although to a lower degree than for *xyr1*. The expression of the coactivator gene *ace2* resembles that of *xyr1* in being inducible by lactose and also requiring CRE1 for full induction. The regulation of

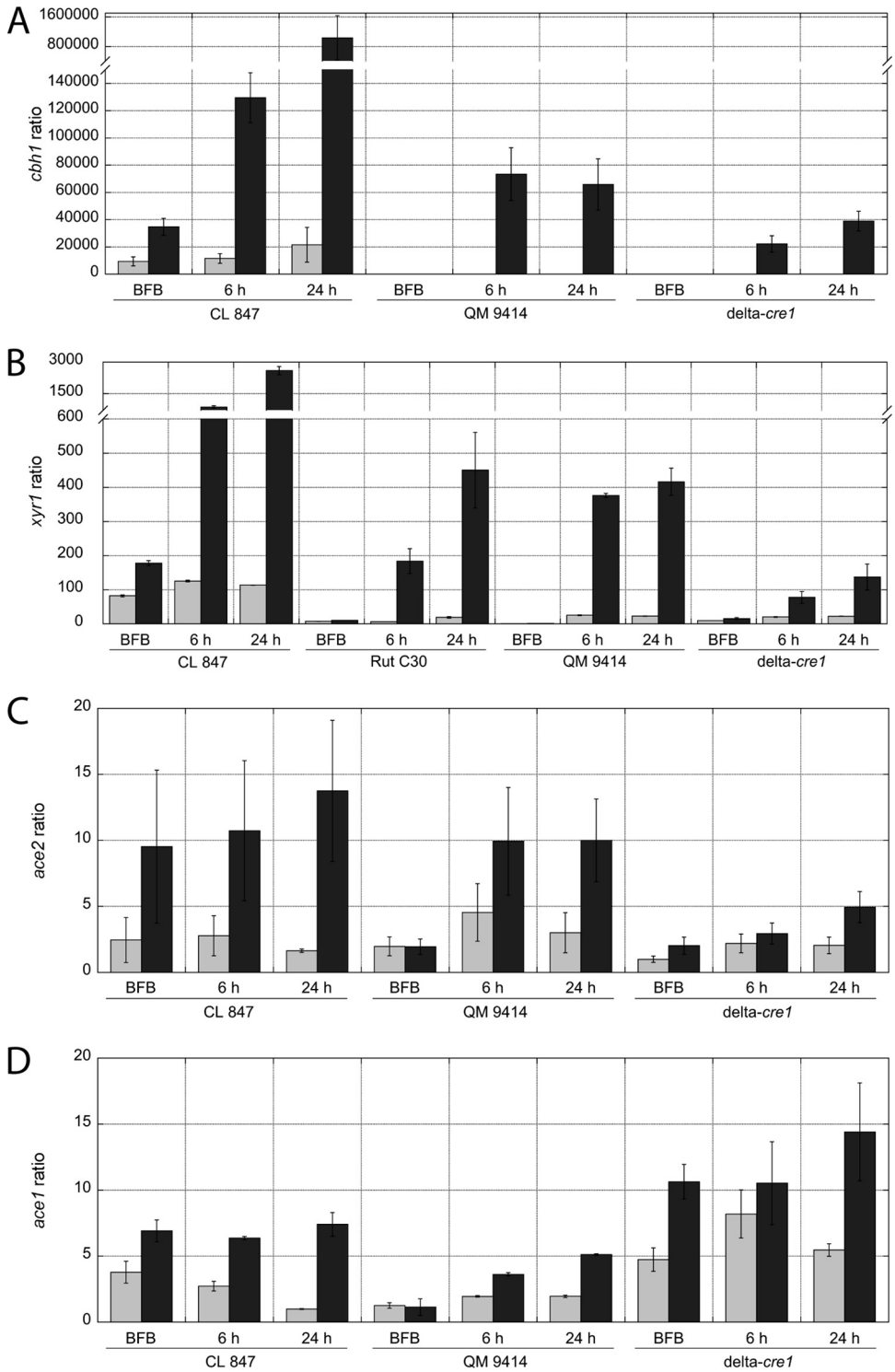

FIG. 6. The expression of cellulase transcriptional regulator genes is altered in the hyperproducing strain CL847. The ratios of the expression of *cbh1* (A), *xyr1* (B), *ace2* (C), and *ace1* (D) transcripts to that of the steady-state *gpd1* reference gene were calculated in *T. reesei* CL847 (and in *T. reesei* RUT C30 [B]) fed with glucose (shaded bars) or with lactose (filled bars) after a 25-h growth phase on glucose. Ratios before (BFB) and 6 h and 24 h after the beginning of the fed-batch phase are shown. Relevant data from Table S2 in the supplemental material and from Fig. 1, 2, and 4 are replotted here for better comparisons.

ace1 expression has been reported previously to be slightly enhanced in the presence of cellulose or sophorose (3), whereas contradictory data on its possible regulation by CCR have been published (3, 19). Here we show—in support of the latter authors—that both basal expression of *ace1* and its induction by lactose are indeed strongly repressed by CRE1. All these findings, as well as data about the effect of galactose on *xyr1* induction (see below), are summarized in Fig. 7.

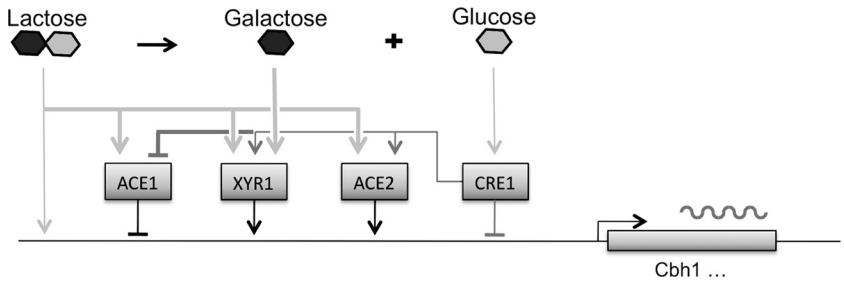

FIG. 7. Regulatory network of cellulase gene expression on lactose. New regulatory relationships described in this work are indicated by bold lines, while the network described previously is indicated by thin lines.

The induction of cellulase gene expression by lactose has recently been shown to be dependent both on the operation of the Leloir pathway and on that of the alternative reductive pathway of D-galactose metabolism (16, 29, 30). Interestingly, the present study shows that lactose induction of *xyr1* was completely independent of galactose metabolism and could also be achieved by D-galactose. The mechanism by which *xyr1* responds to lactose and D-galactose remains unclear. Lactose is not taken up by *T. reesei* (31, 32), and thus, its effect, even in the absence of D-galactose metabolism, must be due to a signaling process. Brunner et al. have recently identified a gene encoding a G protein-coupled receptor from *Trichoderma atroviride* whose silencing resulted—among other features—in a drastic decrease in the ability of this fungus to grow on β-linked galactooligosaccharides (7). An orthologue of this gene is present in *T. reesei* (B. Seiboth and S. Zeilinger, unpublished data) and is expressed under cellulase-forming conditions (expressed sequence tag [EST] clone CB901961) (11). The possible role of this receptor in the lactose induction of *xyr1* is currently under investigation in our laboratory. From a biological perspective, these data show that the presence of lactose (or, in the natural habitat of *T. reesei*, more likely β-galactosides derived from plant cell wall polymers) is a major physiological elicitor for *T. reesei* to shift its transcriptional response toward the degradation of cellulose and hemicelluloses. In support of this hypothesis, we have recently found that XYR1 also regulates L-arabinose and arabinan metabolism in *T. reesei* (1).

There is currently huge interest in the further improvement of cellulase production in existing *T. reesei* mutant strains for second-generation biofuel production (16, 18). We hypothesized that the higher-producing mutants may have also undergone alterations in their transcriptional regulation of the TFs that govern cellulase gene expression. Therefore, we have compared the regulation of *xyr1* in the highest producer strain for which data have been published, *T. reesei* CL847 (9), with that in its obvious parent strain, RUT C30 (10, 23). Under the conditions used in this work, the formation of extracellular protein by strain CL847 on lactose occurred at a ~15-fold-higher rate than that for strain QM 9414. This correlates well with the ~15-fold-increased abundance of the *cbh1* transcript. These data are consistent with the assumption that gene expression may indeed be a major limiting step for cellulase biosynthesis in the parental strain. Consistent with its role as the major transcriptional regulator of cellulase gene expression, we found strongly increased basal expression of *xyr1* in strain CL847, which was further induced by lactose to the approximately 6-fold amplitude similar to the one observed in strain QM 9414. Since the *xyr1* locus in CL847 is perfectly wild type, this increase in gene expression must be due to improved function of the transcriptional machinery required for *xyr1* expression.

The basal expression of *ace2* was not significantly altered in strain CL847, and the inducible level was the same as that in strain QM 9414. This indicates that the lack of CRE1 function, which seems to be required for *ace2* gene expression, as indicated by the lower expression levels in the Δ*cre1* mutant, has been overcome during the breeding of CL847. While these data suggest that *ace2* expression is not limiting for cellulase induction on lactose, they nevertheless show that wild-type expression levels appear to be necessary for the formation of high levels of cellulase.

Expression of *ace1*—even though it is a repressor of cellulase formation—was also increased in the mutant strain CL847. However, *ace1* is subject to CRE1-dependent CCR, and these data therefore must be viewed against the expression levels in the Δ*cre1* strain. The comparison reveals that the basal expression level of *ace1* in CL847 is lower than that in the Δ*cre1* strain and decreases during the glucose feed. The approximate doubling of this level during the lactose feed is conserved, however. We therefore conclude that carbon catabolite derepression of *ace1* has partially reverted in CL847, leading to a lower concentration of this repressor under cellulase-producing conditions.

Taken together, our data strongly suggest that lactose signaling is a trigger for the cellulase transcriptional machinery and that the limiting points in this response reside in the transcription components regulating cellulase gene expression. These data are also in agreement with the recently published genome sequence of a cellulase-producing mutant lineage of *T. reesei*, which revealed that the majority of the affected loci encode formerly unknown transcription factors, proteins acting on mRNA stability, and components of nuclear traffic (18). Mach-Aigner et al. have recently hypothesized that posttranslational modification of XYR1 would be the major mechanism governing cellulase gene expression (19). While we have not specifically looked into this, the present findings of reduced *xyr1* but increased *cbh1* transcription in the Δ*cre1* strain would be consistent with the operation of posttranslational modification of XYR1. Nevertheless, our data show clearly that the expression of *xyr1*, *ace1*, and *ace2* has been significantly altered in the hyperproducer CL847, suggesting that their wild-type expression was insufficient for hyperproduction. Identification

of the proteins and genes responsible for the mechanisms observed in this paper may result in a major breakthrough in the understanding of cellulase formation and may offer a straightforward means for its improvement.

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